Close Integration between Theory and Experiment: Simulations and Chemical Probing Experiments of Molecular Switches

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A riboswitch is an RNA-based molecular switch that plays an important role in regulating bacterial metabolism. This switch senses the presence of a small molecule with high specificity and makes a go/no-go decision for gene expression based on the concentration of the small molecule. In the case of the S-adenosylmethionine (SAM) riboswitch, the riboswitch is embedded in the messenger RNA (mRNA) upstream of the coding region for the enzyme SAM synthetase, which synthesizes SAM. In absence of SAM, a terminator helix forms in the RNA, turning gene expression off and preventing the production of SAM synthetase (Fig. 1). In the presence of SAM, the RNA folds into a different structure that does not contain the terminator helix, allowing production of SAM synthetase. Thus, a single sequence can have two mutually exclusive structures. High concentrations of ligand shift the equilibrium towards the aptamer structure; low concentrations produce a competing structure called the expression platform structure. Using a combination of molecular dynamics (MD) simulations and biochemical probing experiments, we show that divalent ions play a key role in the mechanism of the SAM riboswitch.

RNA molecules are similar to proteins in the sense that they undergo an analogous folding process, participate in conformational changes and catalyze various chemical reactions. One important difference is that the folding and function of RNA molecules is almost always very sensitive to magnesium ions. This is largely a result of the highly charged backbone of the RNA molecule. The exact mechanism of magnesium action is not well understood at the atomistic level. In particular, each magnesium ion is thought to couple with RNA using one of two interaction modes: (1) site-specific binding, or (2) participation in a diffuse magnesium cloud surrounding the RNA.

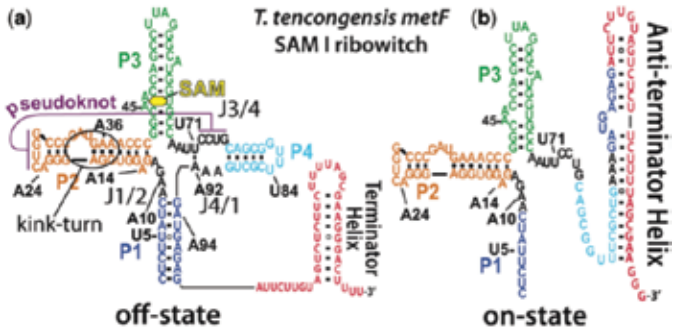

Fig. 1. Secondary structure of the SAM riboswitch RNA molecule. (a) Off-state is formed in the presence of SAM. Terminator helix prevents gene expression. (b) On-state is formed in the absence of SAM. The anti-terminator helix, located in the expression platform domain, precludes formation of the terminator, allowing gene expression.

To elucidate the magnesium action mechanism, we first performed an extensive series of explicit solvent MD simulations of the SAM riboswitch with total sampling of 20 μs [1]. Ten simulations of 2 μs each were performed using a periodic box of water (length of side = 100 Å). Simulations were performed with magnesium concentrations of 0 mM, 1 mM, and 10 mM. A key prediction resulting from this study is that one particular magnesium ion is chelated, interacting directly with the nonbridging phosphate oxygens of residues A10 and U64 in the X-ray structure solved by Batey and co-workers [2] (A10 and U71 in residue numbering of the native wild type SAM riboswitch) (Fig. 2). This was the only magnesium ion site specifically bound to the RNA for the entire simulation.

To test this prediction, we used a biochemical technique called nucleotide analog interference mapping (NAIM). Here, riboswitch RNAs are synthesized that contain modifications at random residues (approximately one modified residue in each RNA molecule). In this set of experiments, phosphothioate modifications were incorporated, replacing a nonbridging phosphate oxygen with a sulfur, which significantly reduces the affinity of magnesium to the RNA in a site-specific manner. The NAIM method requires an assay with which one can measure the effect of the modification. We used an assay that measures switching between the aptamer and expression platform structures. In this assay, the riboswitch is divided into two pieces: the aptamer and a portion of the expression platform. Here, the expression platform strand is an RNA/DNA chimera of 2'-O-methyl RNA and DNA residues. Formation of the expression platform helix creates sites for RNase H cleavage, allowing us to measure the amount of helix formation using RNase H digestion. In the experiments, the aptamer is first folded. Next, the expression platform strand is added. In the absence of magnesium, this strand easily invades the aptamer resulting in formation of the expression platform. In the presence of magnesium, the aptamer is stabilized, preventing formation of the expression platform helix. By comparing wild type and modified RNAs, we are able to determine specific residues, that, when modified, interfere with aptamer stabilization. Because the phosphothioate modification blocks site-specific magnesium binding, the assay reveals specific magnesium chelation sites on the RNA (Fig. 3). We find that this occurs at only four

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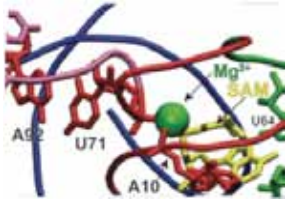

Fig. 2. MD simulations predict a site-specifically bound, chelated magnesium ion (green) connecting riboswitch residues A10 and U64 (A10 and U71 in residue numbering of the native wild type SAM riboswitch).

sites, including two that we predicted with our simulations: A10 and U64 (A10 and U71 in residue numbering of the native wild type SAM riboswitch) [3].

In our simulations, increasing the magnesium concentration slows fluctuations in the RNA. This result is consistent with a set of chemical probing experiments we performed on the riboswitch [3]. Here, we used selective 2'-hydroxyl acylation by primer extension (SHAPE) to measure the backbone mobility of each residue under a wide variety of magnesium and SAM concentrations. We find that the addition of magnesium substantially reduces the RNA mobility.

Interestingly, our experimental study also shows that magnesium and SAM work together to control riboswitch function. In particular, for correct riboswitch operation the addition of SAM needs to produce the aptamer structure and preclude formation of the expression platform helix. However, we find that this does not occur in the presence of SAM alone, even at high concentrations of SAM. Addition of magnesium as well as SAM is required for formation of the fully collapsed aptamer structure. Similarly, magnesium alone cannot produce the fully collapsed aptamer. Some level of SAM is required in addition to the magnesium.

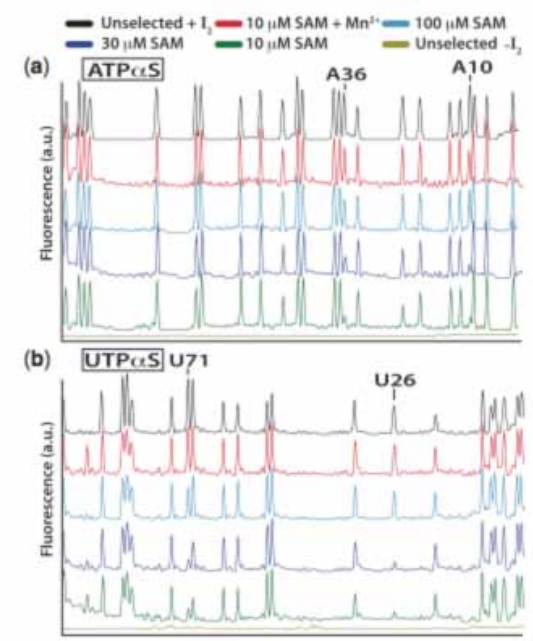

Fig. 3. NAIM shows that a chelated magnesium ion exists near riboswitch residues A10 and U64 (A10 and U71 in residue numbering of the native wild type SAM riboswitch).

When we performed SAM and magnesium titrations in the context of our two-piece switch assay, we discovered that magnesium has the effect of maintaining switch polarity. That is, in the absence of SAM, higher concentrations of magnesium stabilize the expression platform helix; in the presence of SAM, higher concentrations of magnesium stabilize the aptamer structure.

Rather than a few site-specifically bound ions surrounded by a continuum of diffuse ions, we observe a layer of coordinated magnesium that is transiently bound but strongly coupled to the RNA. After measuring diffusion of magnesium ions as a function of distance from the RNA, four categories of ions emerged: (1) free ions, unperturbed by the RNA; (2) diffuse ions with high diffusion coefficients; (3) site-specifically bound ions with near-zero diffusion; and (4) a class of ions called outer-sphere ions with intermediate levels of diffusion (Fig. 4). Surprisingly, outer-sphere ions comprised 80% of the magnesium ions in the simulation. These outer-sphere ions are energetically frustrated and exhibit glass-like behavior, hopping from site to site within the core of the RNA structure and on the surface of the RNA structure. They retain their inner-sphere waters and are separated from the RNA by a single hydration layer. We see that these ions fluctuate on time scales comparable to the fluctuations of the riboswitch and couple to the riboswitch. Their diffusion is two orders of magnitude lower than the bulk magnesium.

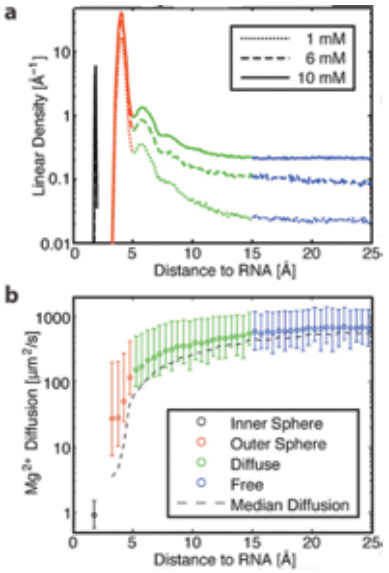

Fig. 4. Explicit solvent MD simulations (20 μs total sampling) reveal four categories of magnesium ions: inner sphere (site-specifically bound), outer sphere, diffuse, and free. The outersphere ions constitute 80% of the magnesium ions. These ions are glass-like, hopping from site to site within the RNA due to energetic frustration.

[1] Hayes, R.L. et al., *J Am Chem Soc* **134** (29) 12043 (2012).

[2] Stoddard C.D. et al., *Structure* 18(7) 787 (2010).

[3] Hennelly, S.P. Novikova, I.V., and Sanbonmatsu K.Y., "The Expression Platform and the Aptamer: Cooperativeity between Mg2+ and Ligand in the SAM-I Roboswithch," *Nculeic Acids Res,* in print (2012).